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The development of adipocytes in primary stromal-vascular culture of fetal pig adipose tissue

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Summary. Primary cultures of stromal-vascular cells of adipose tissue from fetuses at 70 and 110 days of gestation were evaluated as potential model systems for studies of fetal adipocyte differentiation and proliferation. In the cultures, fat cells developed as very discrete clusters. Fat cell cluster development was dependent on initial cell density and time. Histochemical analysis for NADP-dependent dehydrogenases revealed an age of donor effect. Similar levels of enzymes (malate and glucose-6-phosphate dehydrogenase) were apparent in fat cell clusters and stromal cells in cultures of cells from fetuses at 70 days of gestation. These enzymes were only present in fat cell clusters in cultures of cells from fetuses at 110 days of gestation. The distribution of histochemically detectable esterase activity was dependent on the cell density at time of analysis. In areas of high cell density, esterase was restricted to fat cell clusters whereas, both stromal cells and fat cells were esterase reactive in areas of low cell density. Omitting PMS from the dehydrogenase media revealed differences in enzyme reactions of cells grown on collagen-coated and uncoated glass surfaces. These studies demonstrate that primary cultures of stromal-vascular cells from 110-day-old fetuses would be a useful system to identify factors involved in adipocyte proliferation and differentiation.

Key words: Adipocyte – Primary culture – Fetal pig – Histochemistry

Primary stromal-vascular cultures of adipose tissue have been used to determine the presence of preadipocytes in mature adipose depots (Van and Roncari 1978; Bjorntorp et al. 1980). In primary cultures, adipocytes develop and acquire many characteristics of mature adipocytes "in vivo" (Van and Roncari 1978; Bjorntorp et al. 1980). There are no reports on cultures of stromal-vascular cells from fetal adipose tissue.

Subcutaneous fetal pig adipocytes have several unique characteristics. These cells develop early in fetal life, but hypertrophy very slowly (Hausman 1978). Throughout fetal life, they remain multilocular and are arranged as tight clusters of cells (Hausman 1978). The size of fetal pig adipocytes can be altered by changes in maternal and fetal hormonal and metabolic profiles (Hausman et al. 1981; Hausman et al. 1982). However in these instances (Hausman et al. 1981, 1982) the cluster arrangement of adipocytes was persistent despite changes in cell size and histochemistry. To determine if there are traits that are inherent to fetal adipose tissue cells, such as the cluster arrangement, we utilized the primary cell culture system. Histochemistry was used to localize enzyme activities and lipid since it was assumed that not all cultured cells would differentiate.

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Materials and methods

Cell culture techniques. Cells were obtained from 70, 90 and 110 d fetuses from crossbred sows. Two fetuses from each of 3 sows at 70 d of gestation, 1 sow at 90 d and 3 sows at 110 d were utilized for cell culture experiments (i.e., a total of 14 fetuses representing 3 ages). At the respective gestational ages, sows were anesthetized and the uterus exposed and fetuses removed and sacrificed. Fetuses were disinfected with iodine and alcohol and dorsal subcutaneous adipose tissue was removed by sterile dissection.

Adipose tissues were finely minced and incubated with 5 ml/g of tissue of the digestion buffer described by Bjorntorp et al. (1979). After 45 min of incubation at 37° C in a shaking water bath, an equal volume of buffer (room temp.) without enzymes was added to the digestion flask. Flask contents were mixed and filtered through nylon (Tetko, Elmsford, NY) with 275 μm and 20 μm mesh openings to remove undigested tissue and large cell aggregates. The screening procedure is most conveniently performed using Millipore filter holder (# SX0002500) with 25 mm diecut circular screens and a 50 ml syringe as a funnel. The suspended cells were centrifuged for 15 min at $500 \times g$. This separates the cells into a floating fat cell layer and a pellet of preadipocytes and stromal-vascular cells. The fractions were separated and washed with 40 ml of growth media, centrifuged and resuspended.

Aliquots of the stromal-vascular fraction were removed, stained with Rappaport's stain and counted on a hemocytometer. Stromal-vascular cells were seeded on 1.5 coverslips at densities 1×10^4, 0.5×10^4, 0.25×10^4 and 0.125×10^4 cells/cm^2. Before seeding, coverslips were placed in tissue culture well multiplates.

For coating coverslips, a cold, neutral collagen solution

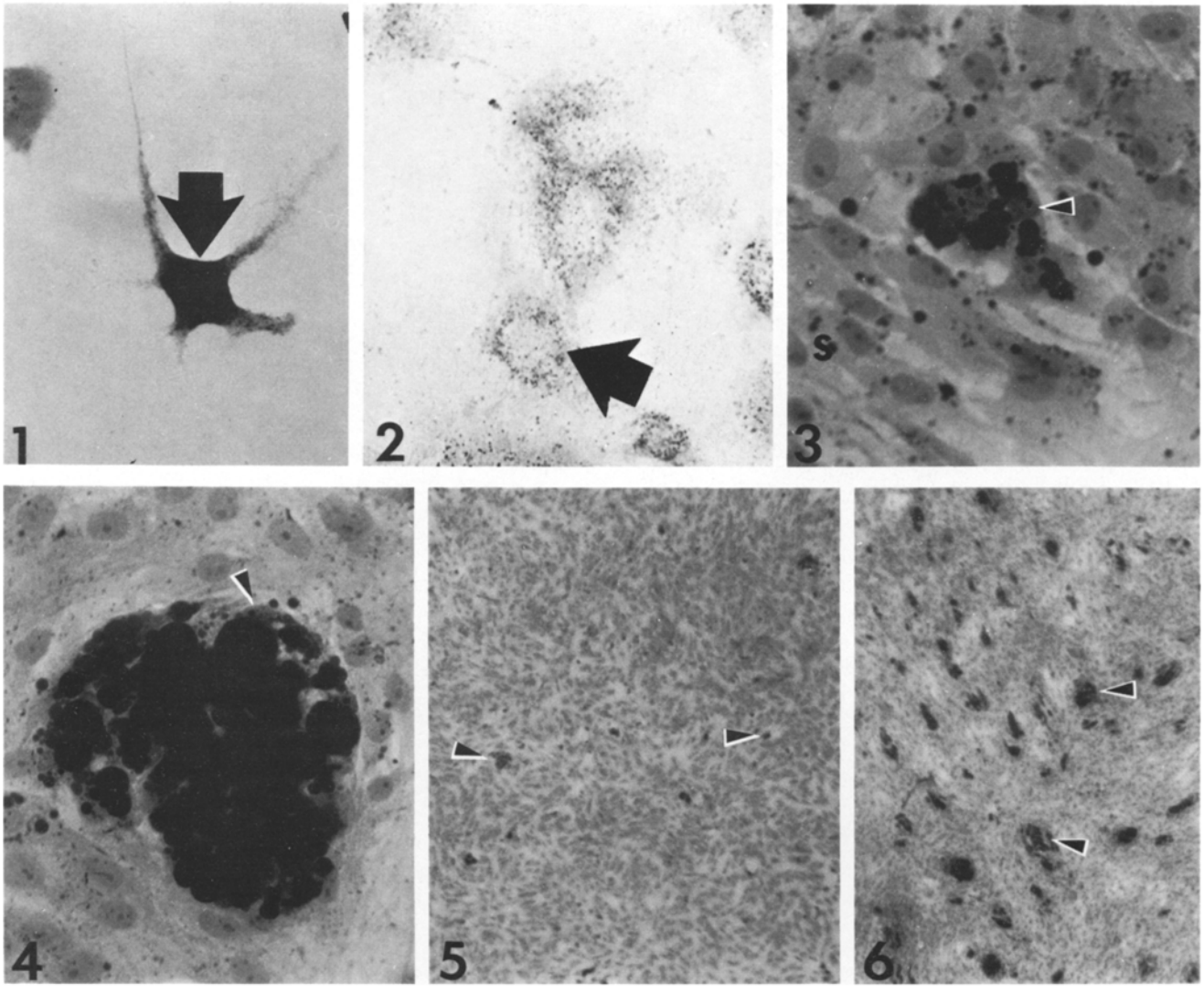

Fig. 1. NADP-dependent malate dehydrogenase (MDH) activity in culture of cells from a 110-day-old fetus. Enzyme activity is located throughout the cytoplasm of this stromal cell (*arrow*). This is a 19-day-old culture seeded at 0.125×10^4 cells/cm. × 350

Fig. 2. NAD-dependent MDH in culture of cells from a 70-day-old fetus. Enzyme activity is present as discrete, small areas in the cytoplasm (*arrow*) of these stromal cells. This is a 6-day-old culture seeded at 0.5×10^4 cells/cm. × 350

Fig. 3. Fat cell cluster (*arrow*) development in a 6-day-old culture of cells from a 70-day-old fetus. Nuclei of fat cells (*arrow*) are small and darkly stained in contrast to stromal (*S*) cell nuclei. Oil red 0 and Harris Hematoxylin (HH). × 350

Fig. 4. Fat cell development in a 10-day-old culture of cells from a 70-day-old fetus. Note the increase in size and number of adipocytes (*arrows*) in the clusters between 6 days (Fig. 3) and 10 days. Oil red 0 and HH. × 350

Fig. 5. Fat cell cluster (*arrows*) development in a 6-day-old culture of cells from a 70-day-old fetus. Oil red 0 and HH. × 20

Fig. 6. Fat cell cluster (*arrows*) development in a 10-day-old culture of cells from a 70-day-old fetus. Note the increase in size and number of cell clusters (*arrows*) between 6 (Fig. 5) and 10 days in culture. Oil red 0 and HH. × 20

was prepared by a modification of the method of Kleinman et al. (1979). Collagen was prepared from rat tail tendons and purified by NaCl precipitation and extensive dialysis. Lyophillized collagen was then dissolved in 0.01 N HCl (1 mg/ml). Coverslips were soaked in a warm 10% NaOH solution for several hours, extensively rinsed and dried. Clean coverslips were then dipped in the collagen solution, dried and autoclaved.

Cells were cultured at 37° C in humidified 5% CO_2 atmosphere. Growth media consisted of Media 199 with Earles salts (Gibco), glucose at a final concentration of 5 mM, 40 mg/l gentamicin sulfate, 2 mg/l Fungizone® (Gibco), 2% fetal calf serum, 2% horse serum and 10% porcine serum. Media was changed on alternate days.

Experiments were terminated at various times between 5 and 19 days after initiation of cultures.

Histochemistry. Two to four coverslips were stained or reacted for each of the following enzymes or components from every experiment.

NADP dependent dehydrogenases. Malate (EC 1.1.1.37) and glucose-6-phosphate (EC 1.1.1.49) dehydrogenase activities were determined according to Rieder et al. (1978). Briefly, coverslips were incubated for 15 min at 37° C in the following media: nitro blue tetrazoleum (NBT) 5.0 mM, 50 mM tris-HCl-buffer pH 7.4, 5.0 mM $MgCl_2$, 10 mM glucose-6-phosphate or 100 mM malate, 0.8 mM NADP, 20% (w/v) PVA, 10 mM NaN_3 and 0.32 mM PMS. Phenazine methosulphate (PMS) was eliminated in several experiments so that the particular dehydrogenase and NADPH-tetrazoleum reductase could be simultaneously demonstrated. As a control, the reaction media without substrate was used; a diffuse and generalized light blue color was observed in only high density cultures. After incubation, fixation was for 15 min in cold (4° C) Baker's formalin.

NAD dependent dehydrogenases. Procedures of Rieder et al. (1978) were modified as follows: NAD replaced NADP at the same concentration, the polyvinyl alcohol solution was eliminated and replaced with the same amount of buffer. As a point of validation, reaction product for NAD-MDH was limited to mitochondria (apparently), whereas reaction product for NADP-MDH was located diffusely in the cytoplasm (Figs. 1, 2).

Alpha-glycerol phosphate dehydrogenase (Gly PDH; EC 1.1.1.8) activity was determined as suggested by Chayen et al. (1973). Briefly, coverslips are incubated (37° C, 15 min) in the following media: 50 mM NBT, 0.05 M glycyl glycine buffer (pH 7.8), 0.05 M glycerol-phosphate, 0.8 mM NAD, 20% PVA (w/v), 10 mM NaN_3 and 0.32 mM PMS.

Lipoprotein lipase activity was demonstrated essentially according to Moskowitz and Moskowitz (1965). Briefly, coverslips were incubated for 2 h at 37° C in a 0.1 M tris base (pH 8.6) that contains 0.02 M $CaCl_2$ and 20% PVA (w/v). Following incubation, coverslips are exposed to a 2.0% lead nitrate solution (15 min) and finally to an ammonium sulfide solution for 2 min.

Esterase (EC 3.1.1.1) activity (-naphthol acetate) was done according to Barka and Anderson (1983). Briefly, coverslips were fixed in cold Baker's formalin (15 min), rinsed in a 0.2 M phosphate buffer and incubated for 15 min (room temp) in the following media: 40% 0.15 M sodium phosphate buffer (pH 7.1), 1%-naphthol acetate in acetone, and 3.2% hexa-pararosaniline-solution. Final pH adjustment is to 7.1.

Coverslip preparations were also stained for lipid (oil red 0; Hausman 1981) and with Harris hematoxylin.

Results

Morphology. Adipocytes were arranged as tight clusters surrounded by "stromal cells" (Figs. 3, 4). All adipocytes contained small and densely stained nuclei and the majority of adipocytes were multilocular (Fig. 3).

Both the number of cells in a cluster and cell size increased with age of culture (Figs. 3–6, Table 1). Fetal age from which tissue was derived had little effect on morphological development.

Histochemistry. Unless otherwise stated, results apply to cells grown on coated coverslips. In cultures where the final cell density was very high, esterase activity was generally restricted to fat cell clusters (Fig. 7). In cultures of lower cell densities, many "stromal cells" were also reactive for esterase (Fig. 8). Fetal age from which tissue was derived had little effect on esterase distribution.

Table 1. Fat cell cluster development in cultures of stromal-vascular cells from fetal pig adipose tissue

Fetal age, day	Plating cell density $\times 10^4$	Days in culture	Fat cell cluster number[a]
70	1	6	10
70	1	10	145
70	0.5	6	5
70	0.5	10	61
110	1	10	195
110	0.5	10	46
110	0.25	10	14
110	0.125	10	24

Coverslips were scanned and the area of the highest total cell density was focused in a low magnification field and all fat cell clusters in the entire field were counted

[a] Coverslips stained with oil red 0 were evaluated

Fat cell clusters were reactive for NADP dependent dehydrogenases (MDH and G6PDH), NAD dependent MDH and lipoprotein lipase activity (Table 2). In cultures of cells from 110-day-old fetuses, these enzyme activities were restricted to fat cell clusters (Fig. 9), whereas stromal cells and fat cells were equally reactive in cultures of cells from 90-day and 70-day fetuses (Fig. 10; Table 2). NAD dependent GlyPDH was not demonstrable in stromal cells or fat cells in cultures from any fetal ages studied.

Eliminating PMS from reaction medias for dehydrogenase histochemistry permits the simultaneous demonstration of the particular dehydrogenase and NADPH-tetrazoleum reductase activity. Activity for NADP dependent MDH without PMS was demonstrable only in adipocyte clusters from 110-day-old fetuses (Table 2). When PMS was omitted from G6PDH and NAD dependent MDH media, there was no activity in any cells from any ages studied.

Effect of plating cell density. Fat cell cluster development was dependent on the plating cell density (Table 1). At similar times in culture, low density cultures yielded fewer fat cell clusters (Table 1). When very low cell densities (0.125×10^4) were plated, there were few (Table 1) or no cell clusters even after 19 days in culture. In old cultures (19-day) without fat cell clusters, single cells contained more lipid than cells in small clusters (Figs. 11, 12; Table 2). The lipid in the single, isolated cells was present as a large number of very small droplets (Fig. 11). Lipid droplets in differentiated adipocytes were fewer and much larger (Figs. 3, 4). The single, isolated cells were reactive for dehydrogenase (MDH, G6PDH) throughout the cytosol (Fig. 1). In contrast, stromal cells in high density cultures (same age) were either not reactive (MDH, G6PDH) or reactivity was limited to the Golgi area. Cells in the low density cultures (0.125×10^4) were not reactive for esterase activity.

Effect of growing cells on uncoated coverslips. Fat cell clusters grown on collagen coated and uncoated coverslips were morphologically similar. Clusters grown on uncoated glass (u-glass) were histochemically distinct from adipocytes on coated glass (c-glass) (Table 2). For instance, NAD dependent GlyPDH activity was demonstrable only in clusters

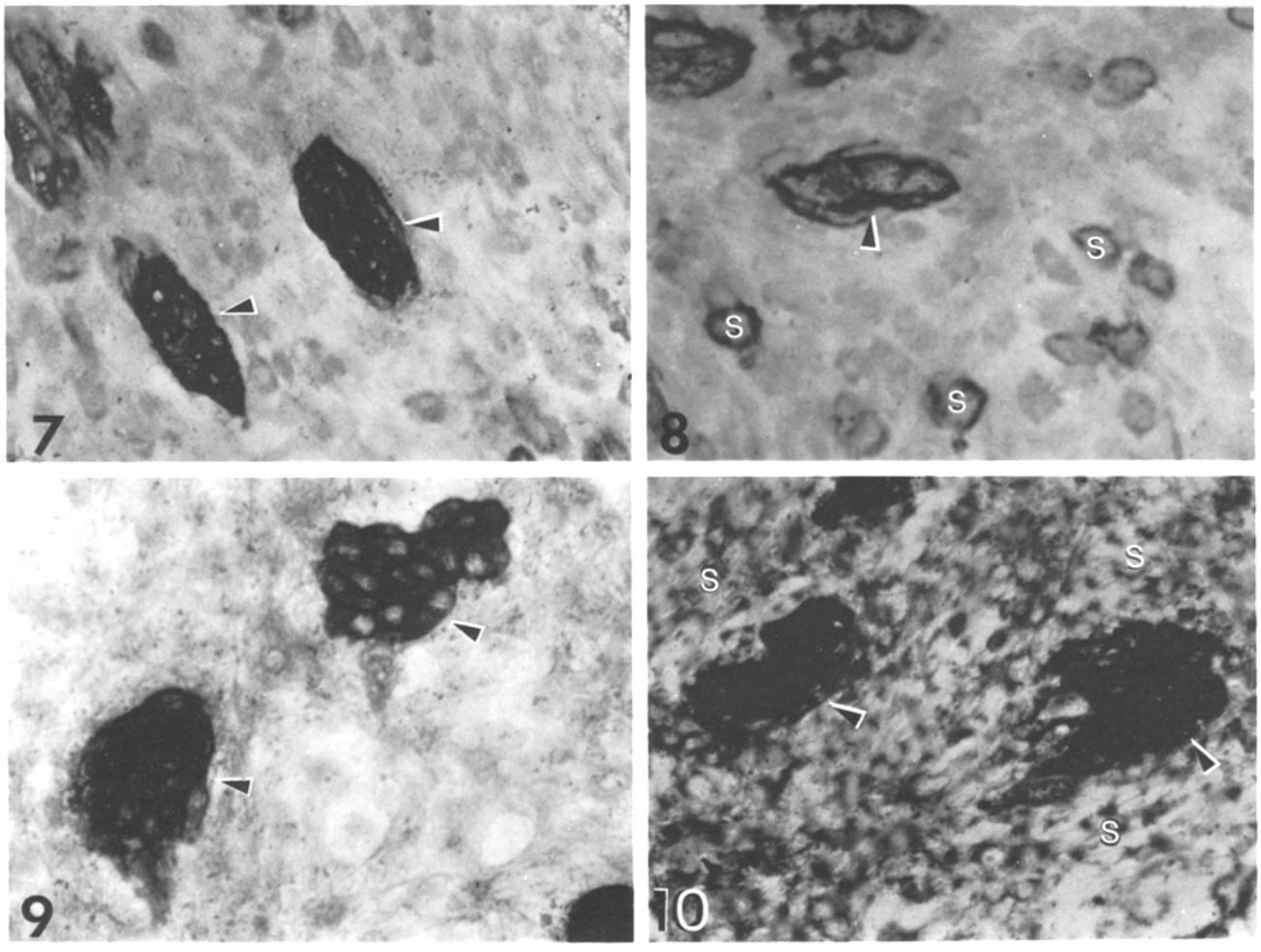

Fig. 7. Esterase activity in fat cell clusters (*arrows*) in 10-day-old culture of cells from a 90-day-old fetus. A final high cell density was associated with enzyme activity restricted to fat cell clusters (*arrows*), × 140

Fig. 8. Esterase activity in 10-day-old culture of cells from a 70-day-old fetus. A low cell density was associated with enzyme activity in clusters (*arrows*) and in some single cells (*S*). × 140

Fig. 9. NADP-dependent MDH activity in cultures (10-day) of cells from a 110-day-old fetus. Enzyme activity is restricted to fat cell clusters (*arrows*). × 140

Fig. 10. NADP-dependent MDH activity in cultures (10 day) of cells from a 70-day-old fetus. Stromal cells (*S*) and fat cell clusters (*arrows*) are reactive. × 140

Table 2. Enzyme and lipid histochemistry of primary cultures of cells from fetal pig adipose tissue[a]

Enzyme	MDH, G6PDH MDH (NAD), LPL		GlyPDH		MDH and G6PDH with no PMS		Lipid		
Coverslip[b]	c-glass		c-glass	u-glass	c-glass	u-glass	c-glass	Surface	
Gestational age, d	70 d	110d	110d	110d	110d	110d	70d[c]	110d[c]	110d[d]
Adipocyte clusters	+	+	−	+	+(MDH)	+	+	+	−
Stromal cells	+	−	−	−	−	+	−	−	+

[a] + = indicates positive reactions whereas − = no reaction
[b] C-glass = collagen coated coverslips, u-glass = uncoated coverslips
[c] Plating cell density was 1×10^4
[d] Plating cell density was 0.125×10^4

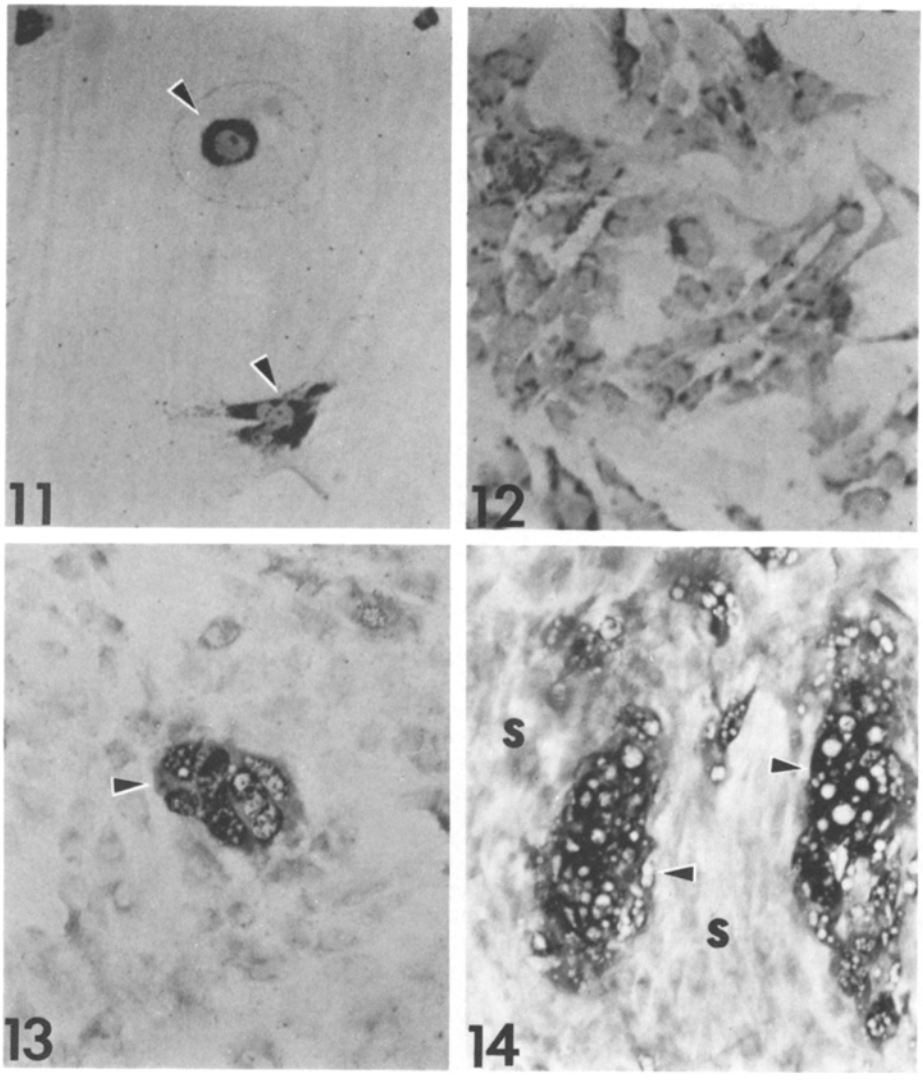

Fig. 11. Lipid staining of single cells in a low density (0.125×10^4) culture (19 day) of cells from a 110-day-old fetus. The single cells contain lipid dispersed as small droplets. Oil red 0 and HH. ×350

Fig. 12. Lipid staining of clumped cells from the same culture as shown in Fig. 11. The clumped cells contain less lipid than is present in single cells (Fig. 11). ×350

Fig. 13. Alpha-glycerol phosphate dehydrogenase activity in a fat cell cluster (*arrow*) grown on uncoated glass. A 10-day culture of cells from a 90-day fetus. ×140

Fig. 14. Glucose-6-phosphate dehydrogenase activity (no PMS) in cultures on uncoated glass. Fat cell clusters (*arrows*) and stromal cells (*S*) are reactive. A 10-day culture of cells from a 90-day fetus. ×140

on u-glass (Fig. 13). When PMS was eliminated from dehydrogenase media (MDH, G6PDH), all the cells (fat cells and stromal cells) on u-glass were reactive (Fig. 14), whereas only fat cell clusters derived from 110-day-old fetuses were reactive when grown on c-glass. No such differences were noted when PMS was used in the dehydrogenase media (MDH and G6PDH). Other histochemical tests were not evaluated on the effect of collagen coating of the glass substrate.

Discussion

These results establish that the tight cluster arrangement of fetal pig adipocytes may be an inherent characteristic of these cells. The apparent dependency of this characteristic on cell confluency and time in culture serves to support the suggestion that the cluster arrangement is an inherent characteristic.

Adipocyte clusters are large and loosely arranged in cultures of 3T3-L1 preadipocytes (Novikoff et al. 1980) and in primary and secondary cultures of mouse, rat and human adipose tissue stromal-vascular cells (Verrando et al. 1981; Van and Roncari 1978). When stromal-vascular cells from either fetal or young rats are cultured with the protocol of the present study, the resultant adipocyte clusters are also large and loosely arranged.[1] Therefore, the tight clusters of differentiated cells may be unique to cultures of fetal pig adipocytes. Recently, rapid and extensive adipocyte differentiation was induced in 3T3-T preadipocytes by heparinized medium containing human plasma (Krawisz and Scott 1982). In the experiments, high cell density and cell to cell contact were not required for adipocyte differentiation (Krawisz and Scott 1982). In the present study, high cell density and cell-to-cell contact were absolute requirements for morphological and biochemical differentiation. A study in which fetal pig stromal-vascular cells are induced with the heparinized human plasma media would further test the inherent character of fetal cells to cluster as they differentiate.

Histochemical data (dehydrogenases and lipoprotein lipase) indicate that stromal cells (in culture) from 70-day and 110-day fetuses are metabolically distinct. In the younger fetuses, stromal cells and adipocytes were equally reactive for enzyme activities whereas only adipocytes from the older fetuses were reactive. This situation may reflect the maturation of stromal cells between 70 days and 110 days in the fetus. For instance, at 110 days, some of the stromal cells may be committed to a fibroblast phenotype,

1 Hausman GJ (1983). Unpublished observations

whereas fewer of the stromal cells would be terminally committed at 70 days. Therefore, cultures of stromal-vascular cells from 70-day fetuses would be more homogeneous and this is true for enzyme distribution. Despite the uniform enzyme distribution, morphological differentiation was only present in cell clusters from 70-day fetuses. Perhaps, the enzyme glycerol-phosphate dehydrogenase is restricted to the adipocyte clusters. This enzyme was restricted to adipocyte clusters when cells were cultured on uncoated glass. Cell growth on coated coverslips may have made the glycerol-phosphate dehydrogenase test uncapable of detecting enzyme activity.

Fat cell clusters from fetal pigs of several ages are reactive for NADH- and NADPH-tetrazoleum reductases (TR) (Hausman and Thomas 1983). In the present study, only fat cells cultured from stromal-vascular cells of 110-day-old fetuses were histochemically reactive for dehydrogenase activity without PMS. This substance (PMS) is a powerful hydrogen-acceptor which rapidly and non-enzymatically takes hydrogen from NADPH and NADH and transfers it to nitro blue tetrazoleum (Chayen et al. 1973). In the absence of PMS, a positive reaction is dependent on both the capability to oxidize either NADPH or NADH to $NADP^+$ and NAD^+ and on the presence of the dehydrogenase. Therefore, both fat cells cultured from stromal-vascular cells of 110-day-old fetuses and fat cells "in vivo" have the capability to oxidize NADPH and NADH (NADPH- and NADH-TR).

Cells cultured on u-glass were reactive for dehydrogenase histochemistry without PMS. Apparently, the collagen coating inhibits the development of the NADPH-TR activity. Conversely, cells grown on u-glass may be more permeable to substrates since GlyPDH activity was only demonstrable in adipocytes on u-glass. A change in membrane permeability may be associated with induction of NADPH-TR activity.

The specificity of esterase staining for fat cell clusters was dependent on cell density of the cultures at time of analysis. Only stromal cells in high density areas were devoid of enzyme activity. Apparently cell-to-cell contact in stromal cells may inhibit esterase activity. In contrast, esterase activity is very obvious in fat cells which are tightly clustered. Therefore, final high cell densities may be important in allowing adipocyte precursor cells and non precursor cells to express their phenotype.

These studies demonstrate that primary cultures of stromal-vascular cells from 110-day-old fetuses could be useful to identify factors involved in adipocyte differentiation and proliferation. Adipocyte clusters are very discrete and can be easily counted across entire coverslips. Enzyme differentiation of the clusters can be monitored histochemically and compared to proliferation of fat cell clusters. Biochemical assays for various enzymes could be done on entire cultures since enzyme activity is apparently restricted to fat cell clusters. In cultures of 3T3-L1 preadipocytes and in primary and secondary cultures of mouse, rat and human adipose tissue stromal-vascular cells, the presence or absence of metabolically heterogeneous adipocytes has not been examined (Novikoff et al. 1980; Verrando et al. 1981; Van and Roncari 1978). If metabolic heterogeneity exists in these other culture systems, the use of biochemical assays for enzyme levels may be misleading since some of the enzyme reactive cells may not contain any lipid. Therefore, one measure of differentiation (enzyme levels) may not correlate with another sign of differentiation (lipid filling).

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